

Steinmetz A, *Mol. Gen. Genet.* 229:238-224, 1991], total sunflower embryo RNA was hybridised with the synthetic primer: 5'-CTCCTGTTCCGGAATTTTGC GTGT-3', [SEQ ID NO:2] whose sequence corresponds to that of the non coding strand of *Ha ds10 G1*, between positions +25 and +48, from the initiation codon. The hybridizations with the primer were carried out at 62°C. The hybrids were extended with AMV reverse transcriptase, for 90 min at 42°C. The extension products were analysed on 6% PAGE sequencing gels, along with sequence reactions produced using the same primer. Initiation sites 1 and 2 (at positions -33 and -25, see SEQ No 1) are functional, and are detected independently using the ribonuclease A protection technique (RNase A, see Figure 3A). A third initiation site (site 3, in position -119 in SEQ No. 1) could not be clearly confirmed with this technique. These initiation sites functionally define the 3' end of the *Ha ds10 G1* gene promoter.

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We modified the RY1 box sited at position -129, verifying by transient expression experiments in sunflower embryos, its functional requirement for the trans-activation of the *Ha ds10 G1* promoter by AB13 type transcription factors (Giraudat J., Hauge BM, Valon C, Smalle J, Parcy F, Goodman HM in *The Plant Cell*: 1251-1261, 1992]. In order to do this we prepared modifications of the *ds10:GUS* fusions constructed for transgenic plants studies (see Example 6.3 and Figure 5). The chimeric genes contained in these two fusions (*ds10F1* and *ds10F2*) are purified as DNA fragments which were subcloned by ligation into pBluescript SK+ (Promega) vector, thus changing the binary vector sequences for smaller ones, more useful for transient expression experiments. We thus obtained the plasmid pSK*ds10F1* using the Sal I – Eco RI fragment (with the chimeric gene obtained from *ds10F1*). In the case of *ds10F2*, the Sph I – Eco RI fragment (from position -125 in *Ha ds10 G1*, to the 3' end of *nos*) was ligated to the complementary fragment (which contains the promoter and 5'-flanking sequences of *Ha ds10 G1*), purified after digestion of pSK*ds10F1* with Sph I and Eco RI, resulting in the pSK*ds10F2* plasmid. Finally, from the pSK*ds10F1* and pSK*ds10F2* plasmids (maps not shown) mutagenised versions were obtained by digestion of their DNA with Sph I, blunting the resulting ends by treatment with T4 DNA polymerase,

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followed by re-ligation of the DNA. We thus obtained plasmids pSKds10F1 Δ RY and pSKds10F2 Δ RY (maps not shown). These plasmids only differ by a nucleotide deletion between positions -126 and -122 of the *Ha ds10 G1* promoter. These changes destroyed the RY1 box present in the ds10F1 and ds10F2 chimeric genes (see Figures 1, 2 and 5), this was verified by the Sanger (dideoxy) method sequencing reactions, using the primer 5'CTCCTGTTCCGGAATTTTGC GTGT3' [SEQ ID NO:2](non coding strand of *Ha ds10G1* between positions +25 and +48).

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sequencing reactions with the Sanger (dideoxy) method, using GUS sequences as the primer: 5'-ACGCGCTTTCCCACCAACGCTG-3'[SEQ ID NO:3].

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The T-DNA in ds10F1, ds10F2, ds10F2 Δ and ds10F3 fusions (Figure 5) was mobilized from *A. tumefaciens* (LBA 4404), obtaining different tobacco transgenic plants with independent integrations of each chimeric gene. These plants were obtained and characterised by standard techniques as described in detail by Coca MA, Almoguera C, Thomas .TL and Jordano J, [in *Plant Molecular Biology*. 31:863-876, 1996]. The expression of *GUS* gene was analyzed both in developing seeds and under normal growth conditions (without exogenous stress) as in seedling tissues, in the latter case the expression changes induced by ABA and dehydration treatments were studied. The seed analysis were carried out with the original transgenic plants (TO), while those of the seedlings used descendants of these plants (T1), segregating for the chimeric genes. Quantitative studies by fluorometric analysis of GUS expression levels and their temporal patterns, as well as qualitative studies which analyzed histochemically the spatial patterns of expression (tissue specificity) were carried out. These studies were carried out as described in detail by Coca MA, Almoguera C, Thomas .TL and Jordano J, [in *Plant Molecular Biology*. 31:863-876, 1996]. In total, the following number (in parenthesis) of tobacco transgenic plants, TO "functional", containing the chimeric genes ds10F1 (14), ds10F2(7), ds10F2 Δ (8) and F3(23) were obtained and analyzed. These plants showed high levels of GUS gene expression in seeds (as a result of the activity of the *Ha ds10 G1* gene promoter and regulatory sequences), as illustrated in Figure 6 (panels A-C). The integration of the different chimeric genes in the transgenic plants' DNA was

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characterized by *Southern* analysis using probes for the coding GUS gene region; PCR amplifications of the sequences close to the ds10::GUS splice, using the 5'-ACGCGCTTTCCCACCAACGCTG-3' [SEQ ID NO:3] (GUS) and 5'-GAGTGAACAgaAATtcCATCACAACAGGG-3' [SEQ ID NO:4] (ds10Eco RI) primers; or by the Kanamycin resistance segregation test (conferred by the *nptII* gene), performed as described in [Jordano J; Almoguera, C; and Thomas, TL, *The Plant Cell* 1:855-866, 1989]. These analysis determined that the TO plant selected for the seed expression studies contained 1 to 5 integrations independent of the corresponding chimeric gene. Figure 6 (joined to this application) illustrates the more relevant results obtained in the study of the expression of the chimeric genes analyzed in transgenic plants. These results are described in detail below.

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Hawkins DJ; Radke SE; and Davies M, in *Science*, 257:72-74, 1992], of storage proteins with compositions rich in specific amino acids [Saalbach I; Pickardt T; Machemehl F, Saalbach G; Schieder O, and Muntz K, in *Molecular and General Genetics* 242:226-236, 1994]; or peptides with antigenic or pharmacological activities [Vandekerckhove J, Van Damme J, Van Lijsebettens M, Botterman J, De Block M, Vandewiele M, De Clercq Leemans J, Van Montagu M, and Krebbers E, in *Bio Technology* 7:929-932, 1989]. These fusions would be carried out and used in an analogous manner to what is described in the publications cited as an example (not exclusive) in each case. To facilitate these possibilities, we have created a plasmid (ds10EC1) that contains an expression cassette including the promoter and the 5'- and 3'-flanking sequences of *Ha ds10 G1* present in ds10F2 Δ (see Figure 5). Between both sequences and by directed mutagenesis [Chen E and Przybila AE, in *Bio Techniques* 17:657-659, 1994], we have added an Eco RI restriction site, which allows the insertion of gene, or corresponding peptide sequences, as mentioned previously (available in other laboratories, or that could be designed or synthesized). The ds10EC1 plasmid was constructed from ds10G1S3 Δ 10.5 (Figure 1). From this plasmid, we amplified the *Ha ds10 G1* sequences between positions -1574 (Sal I) and +98 by PCR; using DNA polymerase Pfu and the primers 5'-ATTAACCCTCACTAAAG-3' [SEQ ID NO:5] (T3) and 5'-

GAGTGAACAgAATtcCATCACAACAGGG-3' [SEQ ID NO:4] (ds10Eco RI). In the latter, the three sequence changes (indicated in lower case letters) introduce the new Eco RI site in the position of the initiation codon. After PCR, a 199 pb (*megaprimer*) DNA fragment is purified, which along with the 5'AATACGACTCACTATAG-3'[SEQ ID NO:6] (T7) primer is used for a second PCR amplification of ds10G1S3 Δ 10.5. The amplified DNA (795 pb) was digested with Eco RI and Sph I. The resulting DNA fragment (125 pb), with the *Ha ds10 G1* sequences between Sph I (-126) and the new Eco RI site, was purified and ligated; replacing in ds10G1S3 the *Ha ds10 G1* (Figure 1) sequences between positions -126 (Sph I) and 1086 (Eco RI). After this step, the PCR amplified sequence was verified by sequencing (Sanger's method) using the T3 primer. Finally, an *Ha ds10 G1* genomic DNA fragment (Figure 1) was inserted in the plasmid obtained in the previous step, with sequences between +1086 (Eco RI) and \approx +3000 (Xba I), obtaining the ds10EC1 *cassette* (Figure 4), cloned in the pBluescript SK+ plasmid. The 3' end of ds10EC1 DNA differs from that of ds10D2 Δ only by 119 additional nucleotides, corresponding to the intron and second exon sequences

Please delete pages 24 and 25 and insert the attached substitute Sequence Listing (new pages 24 through 27).

Please renumber pages 26-28 as pages 29-31.

REMARKS

This amendment is in response to Notice to Comply With Requirements For Patent Applications Containing Nucleotide Sequence and/or Amino Acid Sequence Disclosures mailed April 27, 2001. A copy of the Notice is enclosed. In response to the Notice, applicants have enclosed a paper substitute Sequence Listing and a computer readable Sequence Listing as required by 37 C.F.R. §§1.825 et seq. The application has been amended to delete the existing informal paper Sequence Listing and replace it with the attached formal paper Sequence Listing. The specification has also been amended to insert sequence identifiers. No issue of new matter is raised.